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MECHANISM OF LYSIS OF TARGET CELLS INFECTED WITH MYCOPLASMAS BY NATURAL KILLERS

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KEY WORDS: natural killers; target cells; mycoplasmas; lytic factor.

The important role of natural killer (NK) lymphocytes in antitumor surveillance has been repeatedly confirmed [1, 2, 9, 12]. Despite the large flow of information in the field of the study of NK lymphocytes there is as yet no general agreement on the structures which are the target for these killer cells [3, 13-15]. There is likewise no unanimity on the mechanism by which NK lymphocytes destroy target cells (TC) [10, 16, 18]. Reports were recently published that mycoplasmas, on the one hand, may act as agents stimulating blast transformation of lymphoid cells [6] and, on the other hand, may cause increased sensitivity of TC to the lytic action of NK lymphocytes [4].

This paper confirms observations on more intensive death of mycoplasma-infected TC in the cytotoxic test *in vitro*. However, the cause of the increased mortality of these cells is the cytotoxic action of the mycoplasma, whose weight increases rapidly on account of intensive proliferation of these organisms on effector cells.

EXPERIMENTAL METHOD

The cytotoxic test was carried out by the method described previously [12]; TC were cultured with syngeneic effector cells or with lytic factor in microplates for 48 and 20 h, respectively. The degree of lysis was assessed by the number of living TC remaining on the glass compared with the control. Splenocytes were freed from macrophages by passage through a column containing nylon wadding, as described in [11]. Cell-free lytic factor was obtained by filtering a suspension of syngeneic splenocytes with TC (after incubation for 48 h at 37°C in the ratio of 300 TC to 1.3×10^6 splenocytes in 1 ml of suspension) through a millipore filter with pore diameter of 2.5 μ. Activity of the factor was estimated by the intensity of lysis of L cells (fibroblasts from C3H mice subcultured *in vitro* for a long time). Infectivity of the cells by mycoplasmas was estimated by the difference in incorporation of tritiated uridine and uracil by the cells, i.e., by a method whose principle was described previously [17], and also by means of the dye Hoechst 33258 [5]. If the ratio of labeled uridine to uracil was over 100, the cells were considered to be free from mycoplasmas. Antiserum against mycoplasmas was obtained from a rabbit repeatedly immunized with a suspension of lymphoid cells infected by mycoplasmas. The serum was exhausted by healthy splenocytes, so that in the fluorescence test it caused luminescence only of mycoplasma particles.

EXPERIMENTAL RESULTS

A continuous culture (FC3H3) was obtained from embryonic fibroblasts of C3H mice as a result of spontaneous transformation *in vitro*, and its cells were selected for increased carcinogenicity by triple subculture *in vivo*. After these procedures cells of two tumors were returned to *in vitro* culture (FC3H3V1 and FC3H3V2). The FC3H3V1 cells were accidentally infected in the course of *in vitro* subculture by a mycoplasma (the species was not established), after which the infected subculture was conducted as an independent subline (FC3H3V1.1). As a result of intravenous inoculation of C3H mice with these cells a metastasis was obtained in the lung; its cells, on returning to *in vitro* culture, were found to be mycoplasma-free (FC3H3VM). Data illustrating the purity of the above-mentioned cell cultures, or their in-

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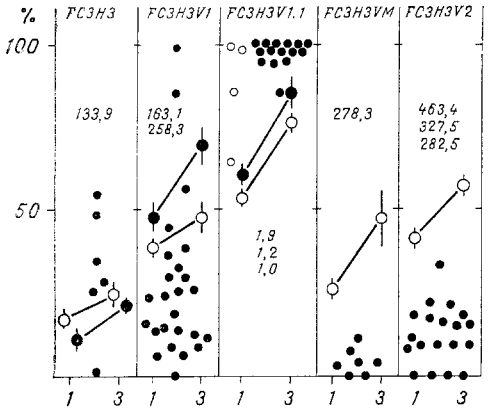

Fig. 1

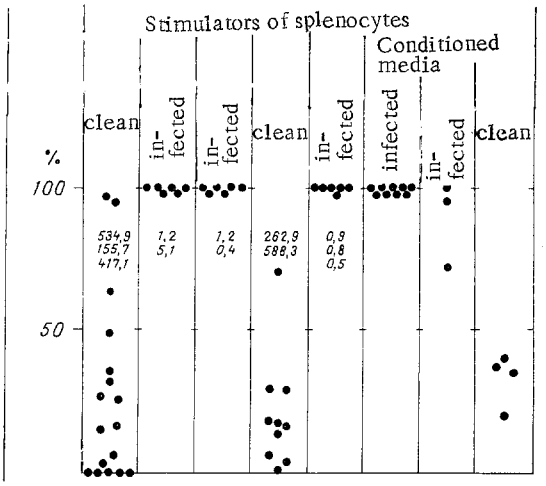

Fig. 2

Fig. 1. Infectivity of cells by mycoplasmas, their sensitivity to lytic action of splenocytes, and ability to stimulate secretion of lytic factor. Numbers in columns give ratio of degree of incorporation of [^{3}H]uridine by cells to degree of incorporation of [^{3}H]uracil (separate experiments). Large circles denote lysis of TC (mean + mean error), small circles denote lysis of cells by separate samples of lytic factor; filled circles denote unfractionated splenocytes; empty circles denote splenocytes passed through column with nylon wadding. Abscissa, concentration of splenocytes ($\times 10^6$/ml); ordinate, percent of lysis of TC.

Fig. 2. Ability of uninfected and mycoplasma-infected cells and of media conditioned by these cells to stimulate splenocytes to secrete lytic factor. Ordinate, % of lysis of L cells. Remainder of legend as to Fig. 1.

fection by mycoplasmas, are given in Fig. 1. The presence of mycoplasmas in FC3H3V1.1 cells was demonstrated not only by the low ratio of the degree of incorporation of labeled uridine by these cells to the degree of incorporation of uracil, but also by the bright cytoplasmic fluorescence of these cells after staining with Hoechst 33258.

Estimation of the sensitivity of the above cultures to the lytic action of syngeneic intact splenocytes showed that FC3H3V1.1 cells exhibited increased sensitivity to these killer cells (Fig. 1). The differences in sensitivity of the cells studied was particularly great in a long-term (48 h) cytotoxic test, which indicated possible activation of lymphocytes during interaction with FC3H3V1.1 cells. This hypothesis was confirmed by data on increased lysis of L cells in the presence of FC3H3V1.1 cells, but not of the other cell lines tested (data not given). However, splenocytes separated from FC3H3V1.1 cells after combined culture for 48 h did not exhibit increased lytic ability; nevertheless, cell-free filtrate of these suspensions did possess this property. In this case filtrates obtained from combined culture of splenocytes with more resistant cells free from mycoplasmas exhibited much lower cytolytic activity (Fig. 1).

Cell-free filtrates of conditioned media from cultures infected with mycoplasmas (posessing infectivity), like infected cells, were able to stimulate the formation of lytic factor (Fig. 2). The lytic factor proved active against all TC: syngeneic, allogeneic, sensitive and resistant to lysis strictly by NK lymphocytes. It was easily fractionated by centrifugation (28,000g) for 30 min into two separately inactive components (residue and supernatant). The residue was held back by a filter with pore diameter of 0.4 μ, whereas the supernatant passed through a filter with pores of 0.22 μ. Cytolytic activity, however, was readily restored to the initial level by mixing these components (Fig. 4). The residue without the supernatant had no cytolytic action even in a four times higher concentration. It is noteworthy that neither component was found in the medium after combined culture of splenocytes with mycoplasma-free TC.

The following facts made the hypothesis likely that the factor (or one of its components) is produced by NK lymphocytes: 1) Freeing of splenocytes from macrophages by passage through columns with nylon wadding affected neither the level of the lytic reactions nor production of the lytic factor (Fig. 1); 2) peritoneal macrophages which, under standard conditions, do

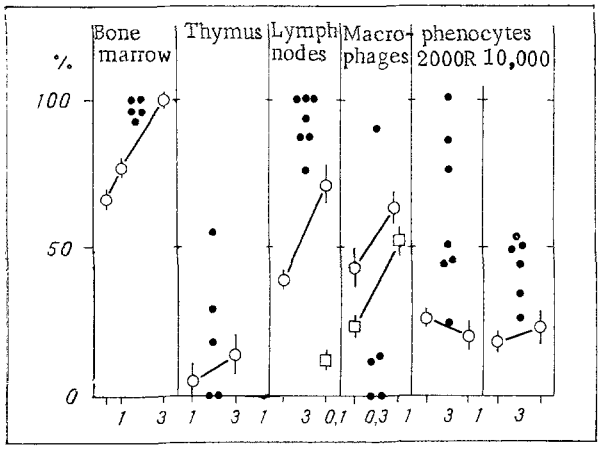

Fig. 3

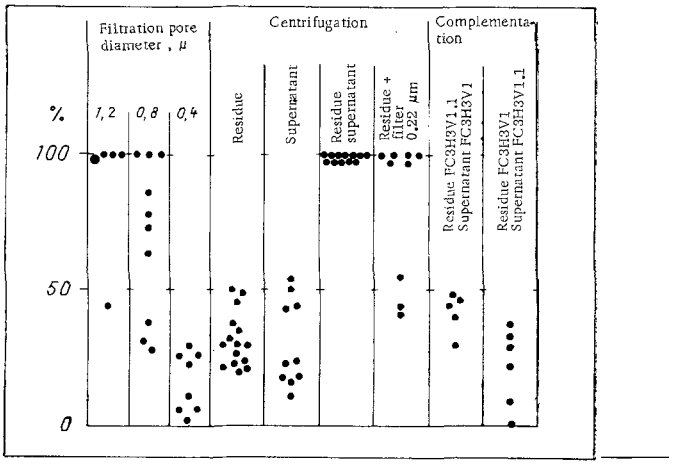

Fig. 4

Fig. 3. Sensitivity of FC3H3V1.1 and FC3H3 cells to lytic action of effectors of various lymphoid organs and of splenocytes killed by irradiation; formation of lytic factor in these tests. Squares denote lysis of FC3H3 cells; large circles — lysis of FC3H3V1.1 cells. Abscissa, concentration of effector cells ($\times 10^6$/ml); vertical axis — percent of lysis of TC. Remainder of legend as to Fig. 1. In all cases effector cells were not fractionated.

Fig. 4. Activity of different components of lytic factor. Ordinate, percent lysis of cells. Remainder of legend as to Fig. 1.

not give rise to lytic factor, had an equally strong cytolytic action on both infected and mycoplasma-free cells (Fig. 3); 3) the high level of lysis of FC3H3V1.1 cells, accompanied by release of lytic factor, was observed only with spleen, bone marrow, and lymph node cells and not with thymus cells (Fig. 3), i.e., it was observed with cells of those organs in which there are many NK lymphocytes [9]; 4) irradiation of splenocytes in a dose of 10,000 R lowered the level of formation of lytic factor and, at the same time, blocked lysis of TC by these cells (Fig. 3); 5) irradiation of TC in the same dose did not affect the level of formation of lytic factor.

Nevertheless, experiments with serum against mycoplasmas yielded data contradicting the original hypothesis. For instance, anti-mycoplasma serum not only completely abolished the strong cytolytic action of splenocytes on TC infected by mycoplasmas, but also significantly inhibited cell lysis by ready-made lytic factor. It was by means of this antiserum (in the indirect fluorescence test) that we identified a residue of lytic factor — consisting of large aggregates of mycoplasmas. The latter also gave intense fluorescence with the dye Hoechst 33258. Millipore filters with a pore diameter of 0.4 μ completely retained these aggregates. In lymphocytes from mycoplasma-infected cell suspensions the antibodies revealed bright punctate fluorescence of mycoplasmas over the whole surface of the cells. The mycoplasmas grew more slowly on thymocytes and on splenocytes irradiated in a dose of 10,000 R, whereas irradiation of the mycoplasmas themselves (of the TC) in the same dose did not prevent their growth on the lymphoid cells.

Mycoplasmas grown on nonlymphoid cells, taken in the same doses, behaved like the residue mentioned above. Production of the humoral part of the lytic factor was not a prerogative either of mycoplasmas or of lymphocytes, for it could be substituted by nontoxic medium conditioned by nonlymphoid, mycoplasma-free cells, taken in high concentrations.

The facts described above thus indicate that long-term cytotoxic tests are unsuitable for use with cultures infected with mycoplasmas.

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MECHANISM OF ACTION OF THE EXOPOLYSACCHARIDE OF *Mycobacterium cyaneum* ON THE LOCAL CELL REACTION IN EXPERIMENTAL *E. coli* INFECTION

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KEY WORDS: mycobacterial polysaccharide; cell reaction; macrophages; phagocytosis; cell enzymes.

Among the substances able to activate the reactions of natural immunity an important place belongs to high-molecular-weight polysaccharides (PS) in the bacterial cell wall. The PS of Gram-negative bacteria have been shown to modify the functional state of cells participating in the immune response [2, 9, 10, 14, 15]. PS of Gram-negative bacteria have received much less study as modulators of defensive reactions.

The aim of the present investigation was to study the effect of PS of *Mycobacterium cyaneum* B-646 on the course of the local cell reaction in experimental *Escherichia coli* infection.

EXPERIMENTAL METHOD

The exopolysaccharide of *M. cyaneum* B-646 (supplied by the All-Union Culture Museum) was obtained by growing the culture on synthetic medium [1]. The culture fluid was deproteinized by Sevag's method, dialyzed, and the PS were precipitated by quaternary ammonium salts [2]. After isolation of the PS from the complex it was lyophilized.

Experiments were carried out on 200 noninbred mice weighing 16-18 g, infected intraperitoneally with *E. coli* (strain 173) in a dose of 5×10^8 bacterial cells [6]. Animals of three groups (50 mice in each group) received PS by intraperitoneal injection in doses of 10 μg (group PS-10), 20 μg (group PS-20), and 50 μg (group PS-50) daily for 3 days before infection. The cell response in the peritoneal exudate (PE) was studied 1-96 h after infection, migration of macrophages and neutrophils, phagocytic activity, number of phagocytic cells, and the intensity of phagocytosis (number of phagocytosed microorganisms) were determined by methods described previously [6, 8], and the phagocytic index was calculated as the product of the parameters of phagocytosis per unit weight of the corresponding cells in PE.

Activity of alkaline and acid phosphatases (AlP and AcP, respectively) by Gomori's method and of peroxidase by the Graham–Knoll method was determined in macrophages and neutrophils,

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